THE TERMINAL OXIDASES OF AZOTOBACTER VINELANDII

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Summary. The terminal respiratory chain of the aerobic nitrogen-fixing bacterium Azotobacter vinelandii was investigated by photochemical action spectra. Terminal cytochrome oxidases a_2, a_1 and o were confirmed as being the terminal oxidases for the physiological substrates NADH and L-malate. TMPD/ascorbate, not giving coupled phosphorylation uses cytochrome a_1 and possibly an o type. DCPIP/ascorbate, giving coupled phosphorylation uses neither cytochrome a_1 nor a_2.

Introduction. Azotobacter vinelandii is an obligate aerobe, nitrogen-fixing organism. It has one of the highest Q_{O_2}'s know. It has a branched terminal respiratory chain (1,2), and three terminal cytochrome oxidases have been shown to be involved in respiration on the basis of difference spectra and CO difference spectra (3). Photochemical action spectra of whole cells oxidising endogenous substrate gave varying results (3,4), but supported the existence of three terminal oxydases. The terminal electron transport chain has been proposed to contain a relatively cyanide insensitive cytochrome $b_1 \longrightarrow$ cytochrome a_2 pathway, and a cytochrome c_4, $c_5 \longrightarrow$ cytochromes a_1, o pathway which is very sensitive to cyanide. (3). Recent studies reported by Ackrell and Jones (6) on oxydative phosphorylation in A. vinelandii suggest the presence of a third phosphorylation site on the cyt. c_4, $c_5 \longrightarrow$ cyt. a_1, o pathway. The terminal respiratory chain can thus be postulated as follows:

Ackrell and Jones (6) found that exogenous c_5/ascorbate or

dichlorophenolindophenol (DCPIP)/ascorbate gave phosphorylation coupled to oxydation, but that tetramethylphenylenediamine (TMPD)/ascorbate or cytochrome c_4/ascorbate did not, indicating a further branching of the chain.

It thus became interest to discover which terminal oxidase was associated with which of these two groups of substrates, from the point of view of further clarification of the A.vinelandii respiratory chain, and also to give more information on the properties of these tree terminal oxidases. One approach to this problem is to investigate photochemical action spectra of A. vinelandii phosphorylating particles in the presence of different substrates. This method will show specifically which terminal oxidase can be used by which substrate.

Method. Chemicals: NADH was obtained from Boehringer und Söhne, Mannheim, DCPIP from Merck, Darmstadt, TMPD and all other chemicals from British Drug House, and were the finest grade obtainable. CO was from Messer-Griesheim (Frankfurt/M., Germany). A. vinelandii NCIB 8660 was grown as described previously.(6). Phosphorylating particles were prepared by a modification of the method described by Ackrell and Jones (6) in that the cell suspension was sonicated in 10 ml aliquots for 30 sec instead of being broken in the French pressure cell. The assay system consisted of 42 mM piperazine ethane sulphonate buffer pH 8,0 8 mM Mg acetate, about 0.30 mg protein and substrate as indicated to a final volume of 3.5 ml. The apparatus for the photochemical action spectra consisted of an anaerobic cuvette with a silvered back surface, equipped with a Clark oxygen electrode and magnetic stirrer. The buffer solutions were placed in the cuvette and gassed with 100% CO until the oxygen concentration in the solution as measured by the oxygen electrode had dropped

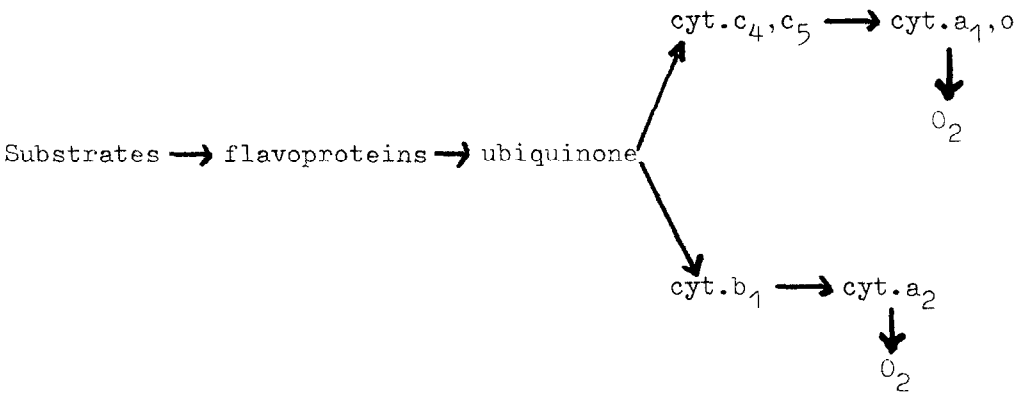

Fig. 1: The terminal respiratory chain in A. vinelandii respiratory particles.

to the required value. The cuvette was then closed and phosphorylating particles followed by substrate introduced into the cuvette by a syringe inserted through a rubber septum. Light intensity was measured with a thermocouple and microameter (Kipp and Zonen, Delft, Holland). Light of different wavelengths was provided from a 1600 W Xenon High Pressure Lamp (Osram XBO 1600) by precision monochromatic interference filters with a transmission half width of 10 nm. Heat protection filters were placed between the monochromatic filters and light source. All the filters were obtained from Jenaer Glaswerk Schott and Gen. Mainz, Germany. The arrangement was described by Diehl et al. (7).

The reactions were started by the addition of substrate and after a constant time interval of reaction in the dark, light of the desired wavelength was shone on the cuvette. The light intensity was kept constant as was the O_2/ CO ratio for the series of experiments required for each action spectrum. The photochemical action spectra were plotted as $L/_{L\ ref.}$ vs.

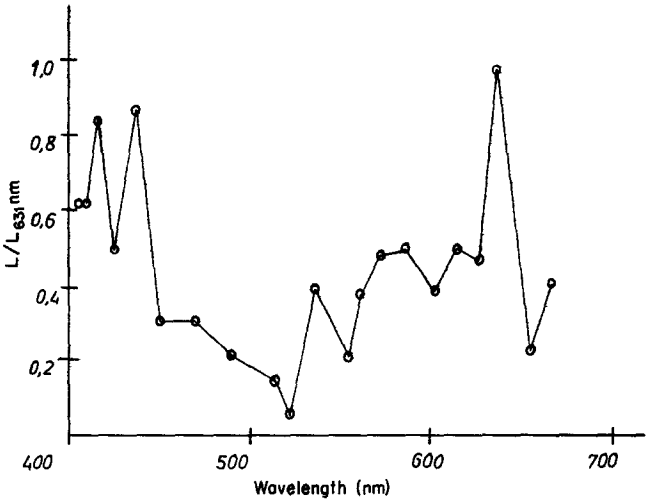

Fig. 2. Photochemical action spectra of NADH oxidase of A. vinelandii phosphorylating membranes. Protein = 0,1 mg/ml; [NADH] = 1 mM; $[O_2]$ = 20% of original, rest displaced with 100% CO. Each point represents the average of at least two determinations; from left to right they indicate the following wavelengths: 407, 411, 417, 424, 436, 450, 470, 490, 515, 521, 533, 555, 560, 572, 583, 602, 615, 626, 637, 655, and 676 nm.

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wavelength, where $L = \frac{1}{i} \frac{\Delta K}{K_{dark}}$ and $\Delta K = K_{light} - K_{dark}$ with $K = \frac{n}{1-n} \cdot \frac{CO}{O_2}$ and $n = \frac{\text{activity rate with CO}}{\text{activity rate without CO}}$ (5).

Results and Discussion. Figure 2 shows the photochemical action spectrum obtained with NADH and phosphorylating particles from A. vinelandii. It is very similar in the visible region to those obtained for whole cells oxidising endogenous substrate (3,4). Similar spectra are obtained with L-malate. As can be seen, all three terminal oxidases are involved in NADH dependent respiration with light relief maximum at 637 nm, characteristic of cyt. a_2, about 583 nm, and about 436 nm characteristic of an a cytochrome, at 570 nm, and about 537 nm characteristic of cyt. o. The small maximum at 615 nm is not yet identified but may be another a type cytochrome.

Figure 3 shows the photochemical action spectrum obtained with TMPD/ascorbate as substrate. There is no light relief at 637 nm

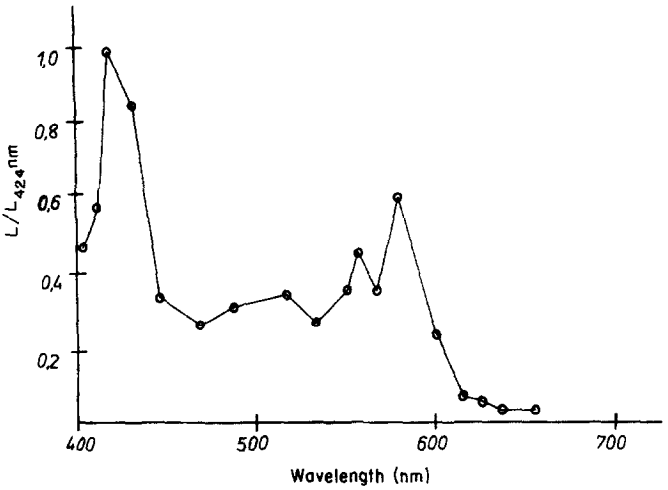

Fig. 3. Photochemical action spectra of Ascorbate-TMPD oxidase of A. vinelandii phosphorylating membranes. Protein = 0,1 mg/ml; Ascorbate-TMPD = 4 mM; $[O_2]$= 20% of original, rest displaced with 100% CO. Each point represents the average of at least two determinations; from left to right they indicate the following wavelengths: 407, 417, 424, 436, 450, 470, 490, 515, 533, 555, 560, 572, 583, 602, 615, 626, 637, and 655 nm.

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indicating that TMPD/ascorbate does not use cyt. $\underline{a}_2$. The maximum obtained about 583 nm and 424 nm are indicative of cyt. $\underline{a}_1$. The maximum at 560 nm is as yet unidentified, but may be an $\underline{o}$ type cytochrome different from the $\underline{o}_{570}$ found with NADH as substrate. Similar results were obtained at a higher O_2/CO ratio. Results with DCPIP/ascorbate were disappointing since this oxidase is the least sensitive to CO. Therefore, it was difficult to obtain inhibition sufficient to give reproducible results. However, we can suggest that neither cyt. $\underline{a}_1$ nor $\underline{a}_2$ are involved, as light relief of the DCPIP/ascorbate oxidase was not obtained reproducibly at wavelengths corresponding to the maximum for these cytochromes. We have since learned that although DCPIP/ascorbate and TMPD/ascorbate oxidases are equally sensitive to KCN, the inhibition is of different kinetic types (C.W. Jones, personal

communication). This gives further evidence that neither $\underline{a}_1$ nor $\underline{a}_2$ are involved in DCPIP/ascorbate oxidation.

From the results obtained, we would suggest that an $\underline{o}$ type

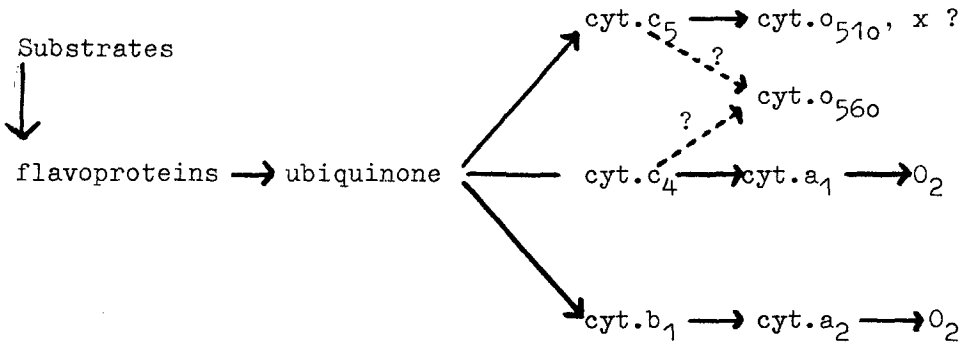

Fig. 4: Suggested terminal respiratory chain in A. vinelandii respiratory particles.

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cytochrome (s) and possibly an unidentified cytochrome, x, are involved as the DCPIP/ascorbate oxidase.

Therefore, if we assume that both DCPIP and TMPD donate their electrons at the cytochrome $\underline{c}$ level, we suggest the terminal electron transport system in A. vinelandii respiratory particles to be as shown in figure 4.

In conlusion it is perhaps interesting to note that on the basis of this work, neither cytochrome $\underline{a}_1$ nor $\underline{a}_2$ would appear to have phosphorylation sites associated with them.

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